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Hum Genet (1995) 95:557–561

ORIGINAL INVESTIGATION

Toshika Okumiya · Satoshi Ishii · Ryoichi Kase
Sachiko Kamei · Hitoshi Sakuraba · Yoshiyuki Suzuki

α-Galactosidase gene mutations in Fabry disease: heterogeneous expressions of mutant enzyme proteins

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Abstract Five point mutations were identified in unrelated Japanese Fabry disease hemizygotes: three new missense mutations, C142Y (^{425} G → A), A156V (^{467} C → T), and L166V (^{496} C → G) in exon 3; one new splice site mutation at the 3′ end of the consensus sequence in exon 4; one previously reported nonsense mutation, W44X (^{131} G → A). C142Y expressed 50% of the normal enzyme protein in COS-1 cells, but catalytic activity was not detected. Both A156V and L166V expressed significant amounts of residual enzyme activity (6.7% and 9.8%) and enzyme proteins (10% each), the latter were more thermolabile at neutral pH than at acid pH, in vitro.

Introduction

Human α-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22; α-Gal) is a lysosomal hydrolase encoded by a gene localized at Xq22. Deficient activity of α-Gal results in Fabry disease causing intralysosomal accumulation of glycosphingolipids, mainly globotriaosylceramide. Patients with the classic form of the disease with early onset show diverse clinical manifestations, such as pain and paresthesia in distal extremities, angiokeratoma, hypohidrosis, corneal opacity, and progressive vasculopathy of the kidney, heart, and central nervous system (Desnick and Bishop 1989). Recently, a new atypical disease with progressive cardiomyopathy, occurring after 50 years of age, has also been recognized (cardiac form; Sakuraba et al. 1990; Nagao et al. 1991; Scheidt et al. 1991; Ishii et al. 1992; Eng et al. 1993).

T. Okumiya · S. Ishii · R. Kase · H. Sakuraba (✉) · Y. Suzuki
Department of Clinical Genetics,
The Tokyo Metropolitan Institute of Medical Science,
3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113, Japan

T. Okumiya · S. Kamei
School of Allied Health Sciences,
Tokyo Medical and Dental University, Tokyo, Japan

Mutant α-Gal gene products in classic Fabry disease have no detectable catalytic activity, and the amount of enzyme protein is low (Koide et al. 1990; Ishii et al. 1992; R. Kase et al. unpublished data). A detectable amount of residual enzyme activity has been reported for the late-onset cardiac form. In this study, we identified four new mutations in Fabry patients, and mutant gene products were further characterized.

Patients

The diagnosis of five Japanese male patients was confirmed by α-Gal assay in peripheral leukocytes and/or cultured lymphoblasts. Their clinical data are summarized in Table 1.

Materials and methods

Cell culture

Lymphoblast cell lines were established by transformation with Epstein-Barr virus, and cultured at 37° C in 5% CO_2 in RPMI-1640

Table 1 Clinical summary of patients examined in this study (*NE*, not examined, *ND*, not detected)

Patient no.	1	2	3[a]	4	5
Sex	Male	Male	Male	Male	Male
Age (year)	14	16	5	25	11
Pain/acroparesthesia	+	+	−	+	+
Hypohidrosis	+	+	−	+	+
Angiokeratoma	−	−	−	+	−
Corneal opactiy	NE	+	−	−	−
Proteinuria	+	−	−	+	−
Cardiac involvement	−	−	−	−	−
α-Gal activity[b]	0.1	ND	0.4	0.4	0.6

[a] His mother had proteinuria, and an electron microscopic study revealed intracellular concentric or lamellar inclusions, in kidney tissues obtained by biopsy
[b] Enzyme activity in lymphoblasts (nmol/h per mg protein). Classic Fabry patients: 0.4 ± 0.2 [21], cardiac Fabry patients: 3.9 ± 0.7 [3], and normal controls: 87.1 ± 32.1 [24] (mean ± SD [*n*])

Table 2 Oligonucleotides used for α-Gal cDNA and genomic DNA amplification, and sequencing

Primer	Sequence[a]	Orientation[b]	Coordinate[c]
1	5′-TGA*GGATCC*TTTATGCTGTCCGGTCAC-3′	+	−25 to −8
2	5′-TGA*GAATTC*TGGTCCAGCAACATCAACAA-3′	−	758 to 778
3	5′-TGA*GAATTC*GTATCTTGGACTGGACATCT-3′	+	722 to 741
4	5′-GCC*GGATCC*TTAAAGTAAGTCTTTTAATGAC-3′	−	1269 to 1290
5	5′-TGA*GAATTC*AATTTATGCTGTCCGGTCA-3′	+	(1153) to (1171)
6	5′-TGA*GAATTC*GGGAGTACCCAATATCTG-3′	−	(1378) to (1395)
7	5′-CCG*GTCGAC*GAAGAGTCTTTGTCAAGGTA-3′	+	(6941) to (6960)
8	5′-TGA*GAATTC*TTGACAACCTGGACTCCC-3′	−	(7731) to (7748)
9	5′-TGC*GGATCC*AGCTGGAAATTCATTTCT-3′	+	(8268) to (8287)
10	5′-GTC*GAATTC*CAGGTGATGGTAGCTT-3′	−	(8578) to (8596)
11	5′-GAGCTCATGGTCTCAGAA-3′	+	220 to 237
12	5′-GCTGACTGGGGAGTAGA-3′	+	478 to 494
13	5′-TACAGCCCAGGCTAAGCC-3′	−	1036 to 1053

[a] Restriction sites (*Bam*HI or *Eco*RI) are underlined
[b] Indicated as sense (+) or antisense (−)
[c] Numbered for the sequences of full-length α-Gal cDNA (Bishop et al. 1988) and the 12-kb gene (Kornreich et al. 1989). Genomic coordinates are given in parentheses

medium (Nissui Pharmaceutical, Tokyo, Japan), supplemented with 10% fetal calf serum (FCS) and antibiotics.

Isolation of poly(A)⁺RNA and genomic DNA

Total RNA was isolated from cultured lymphoblasts by the guanidine isothiocyanate/cesium chloride procedure, and poly(A)⁺RNA was purified with an oligo(dT)-cellulose column (Sambrook et al. 1989). Genomic DNA was isolated from cultured lymphoblasts by the standard method (Aldridge et al. 1984).

Amplificiation and sequencing of reverse-transcribed α-Gal mRNA

Two fragments of α-Gal cDNA, partially overlapping with each other, were synthesized and amplified by the reverse transcription-polymerase chain reaction (RT-PCR), using a GeneAmp Reverse Transcriptase RNA PCR Kit (Perkin Elmer-Cetus, Norwalk, CT, USA). In these reactions, two sets of primers denoted in Table 2 were used: primers 1 and 2 for the 5′ region (fragment A: α-Gal cDNA base −25 to 778); primers 3 and 4 for the 3′ region (fragment B: α-Gal cDNA base 722 to 1290). For reverse transcription, 0.25 μg poly(A)⁺RNA was mixed with 0.75 μ*M* antisense primer, 5 u *Thermus thermophilus* DNA polymerase, 200 μ*M* dNTP, 1 m*M* $MnCl_2$, 10 m*M* TRIS-HCl (pH 8.3), 90 m*M* KCl, in a volume of 20 μl, and the mixture was incubated at 70° C for 10 min. Then, a PCR reaction mixture (80 μl) was added, which contained 0.2 μ*M* sense primer, 2.5 m*M* $MgCl_2$, 5% glycerol, 10 m*M* TRIS-HCl (pH 8.3) 100 m*M* KCl, 0.75 m*M* ethylene glycol-bis(β-aminoethyl ether), N,N,N′,N′-tetraacetic acid, 0.05% Tween 20. The conditions for thermal cycling with a DNA Thermal Cycler (Perkin Elmer-Cetus) were: 35 cycles of denaturing for 1 min at 94° C, annealing and extension for 1 min at 65° C. The RT-PCR products were subcloned into *Bam*HI and *Eco*RI sites of the M13mp18 vector. Nucleotide sequences were determined by the dideoxy chain-termination method (Sanger et al. 1977), with one universal primer and three other primers: primers 11, 12, and 13 (Table 2).

Amplification and sequencing of genomic DNA

For confirmation of the mutations identified in the cDNA fragments, genomic fragments were amplified by PCR, using three sets of primers: primers 5 and 6 for exon 1; primers 7 and 8 for exon 3; primers 9 and 10 for exon 4 (Table 2). The amplified fragments were analyzed by restriction analysis with appropriate restriction enzymes, or by nucleotide sequencing as described above.

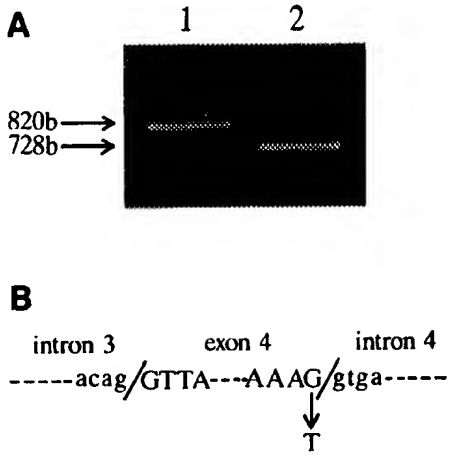

Fig. 1 A, B A single base substitution causing exon 4 skipping in patient 1. A Electrophoretic pattern of α-Gal cDNA. Fragment A, comprising exons 1–4 and a part of exon 5, was amplified by RT-PCR, electrophoresed in a 1.5% agarose gel, and stained with ethidium bromide. *Lane 1* Normal subject, *lane 2* patient 1. B The mutation site of α-Gal genomic DNA in patient 1. A genomic DNA fragment surrounding exon 4 was amplified by PCR, and the nucleotide sequence was determined

Transient expression

The entire wild-type α-Gal cDNA was inserted into the *Eco*RI site of the pCXN2 expression vector (kindly supplied by Dr. J.-i. Miyazaki, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan) to perform a high level expression analysis in COS-1 cells (pCXN2-Gal; Ishii et al. 1993). Each missense-mutant cDNA was digested with appropriate restriction enzymes, and the cDNA fragment containing a single base substitution was inserted into the region corresponding to the digested region in pCXN2-Gal. The mutant plasmids were designated as pCXN2-C142Y (patient 3), pCXN2-A156V (patient 4), and pCXN2-L166V (patient 5). They were subcloned and amplified in *E. coli* (DH-5α). COS-1 cells, cultured at 37° C in 5% CO_2 in Ham's F-10 medium supplemented with 10% FCS and antibiotics, were transfected with 20 μg plasmid DNA per 60-mm dish, by the calcium phosphate-glycerol shock technique (Koide et al. 1990). The cells were harvested after 72 h, and homogenized in distilled water by freezing and thawing. The supernatant obtained after centrifugation at 10000 *g* was used for α-Gal assay and Western blotting.

Table 3 Exonic point mutations in four patients with Fabry disease

Patient	Exon	Mutation	Base substitution	Amino acid substitution	Restriction site	
					Enzyme	Change[a]
2	1	W44X	TGG → TAG	Trp → Stop	*Nhe*I	+
3	3	C142Y	TGC → TAC	Cys → Tyr	*Hha*I	–
4	3	A156V	GCC → GTC	Ala → Val	*Sfa*NI	–
5	3	L166V	CTG → GTG	Leu → Val	*Bgl*II	–

[a] + Site created, – site eliminated

α-Gal assay

α-Gal activity was assayed with an artificial substrate, 4-methylumbelliferyl α-D-galactoside (Nacalai Tesque, Kyoto, Japan), as described previously (Ishii et al. 1994). One unit of enzyme activity was defined as 1 μmol 4-methylumbelliferone released per h at 37° C. Protein concentration was determined by the method of Bradford (1976), using a protein assay kit (Bio-Rad, South Richmond, CA, USA).

Western blotting

Western blotting was performed under the conditions described by Towbin et al. (1979), with an anti-α-Gal antibody raised in rabbits (Ishii et al. 1994). The amount of the expressed proteins was quantitated by means of an image analysis system (Minamikawa-Tachino et al. 1993).

Results

cDNA and genomic DNA sequencing

The fragment A in patient 1, comprising exons 1–4 and a part of exon 5, was smaller in size than that in the normal control (Fig. 1 A). Its sequence analysis revealed a complete skipping of exon 4, and consequently exon 3 was directly connected with exon 5. A PCR-amplified genomic fragment comprising exon 4, however, was normal in size, and a single G-to-T substitution was found at the 3′ end of exon 4 (5′-splice donor site), thereby altering the invariant consensus sequence for splicing (Fig. 1 B). This base substitution was the only abnormality in the amplified genomic fragment.

Single base substitutions were also confirmed for the other patients. The results of cDNA sequencing are summarized in Table 3. These mutations either created a new restriction site or eliminated an existing restriction site; restriction analysis confirmed each mutation. In patient 2, a previously reported nonsense mutation in exon 1 (Sakuraba et al. 1990) was found: a G-to-A substitution in codon 44, generating a stop codon. Three new mutations were found for patients 3, 4, and 5: Cys → Tyr, Ala → Val, and Leu → Val, respectively.

Transient expression of missense mutations

A 60-fold increase in α-Gal activity was achieved by transfection into COS-1 cells with wild-type cDNA, as compared with mock transfection (Fig. 2, upper panel), and the expressed enzyme protein was demonstrated at 46 kDa by immunoblotting (Fig. 2, lower panel). Enzyme protein was expressed at half the normal level for pCXN2-C142Y (patient 3) (50%; Fig. 2, lane 2), but catalytic activity was completely lost. In the other two mutations, pCXN2-A156V (patient 4) and pCXN2-L166V (patient 5), detectable catalytic activity (6.7% and 9.8%, respectively) and enzyme protein (approximately 10% each) were observed (Fig. 2, lanes 4 and 5).

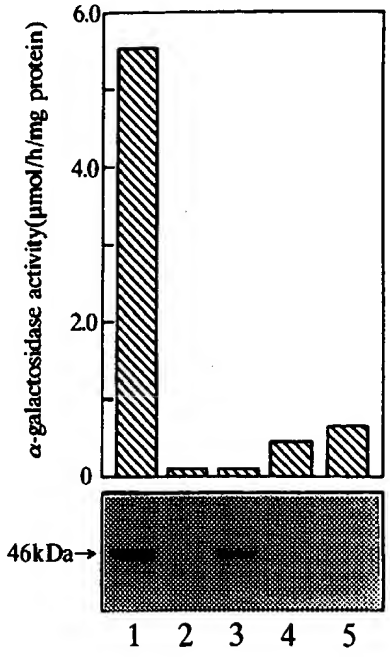

Fig. 2 Transient expression of α-Gal cDNA in COS-1 cells. *Upper panel* Enzyme activity (an average of duplicate determinations). *Lower panel* Western blotting under non-reducing conditions. The enzyme protein was immunologically detected as a band at 46 kDa. *Lane 1* Normal control, *lane 2* mock transfection, *lane 3* patient 3 (C142Y), *lane 4* patient 4 (A156V), *lane 5* patient 5 (L166V)

Thermostability of expressed mutant enzymes

The COS-1 cells transfected with mutant cDNA were homogenized in 2 vol 0.1 *M* citrate/0.2 *M* phosphate buffer, pH 7.0 or 4.6, and incubated for 2 h at 42°C. The expressed enzyme activities of pCXN2-A156V (patient 4) and pCXN2-L166V (patient 5) were markedly unstable, especially at pH 7.0 (Fig. 3). The degree of the residual activities in COS-1 cells was apparently correlated with

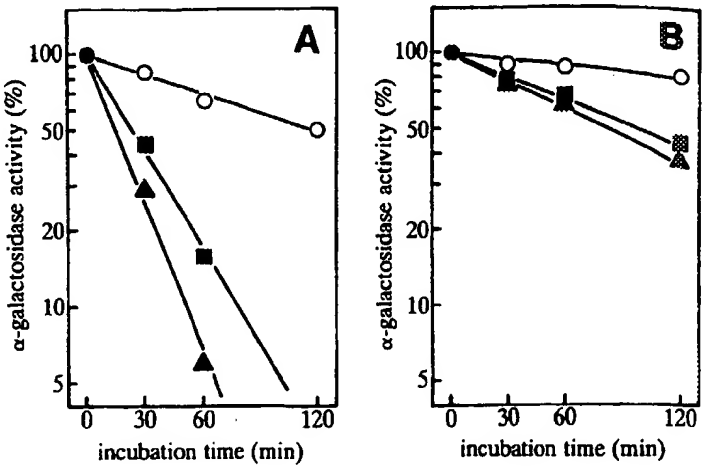

Fig. 3A, B Effects of pH and temperature on the stability of mutant enzymes expressed in COS-1 cells. Crude extract from the cells with wild-type α-Gal cDNA (○), mutant A156V (▲), or mutant L166V (■) was incubated with 2 vol 0.1 *M* citrate/0.2 *M* phosphate buffer at pH 7.0 (**A**), or pH 4.6 (**B**), at 42°C for 2 h. Each value is expressed as a percent of the activity before incubation

that of the thermostability, i.e., the enzyme was more stable in patient 5 than in patient 4.

Discussion

Since the cloning and sequencing of α-Gal cDNA (Calhoun et al. 1985; Bishop et al. 1986), molecular genetic analysis has been carried out for Fabry disease. Mutations are heterogeneous (Sakuraba et al. 1990, 1992; Fukuhara et al 1990; Ishii et al. 1991; Scheidt et al. 1991; Eng et al. 1993), but gross gene rearrangements have been observed in only 5% of the patients analyzed (Bernstein et al. 1989). In most of the other patients, several point mutations causing single amino acid substitutions have been found. However, mutant gene products have been characterized in only a few of them (Koide et al. 1990; Ishii et al. 1992, 1993).

In this study, we identified five point mutations in Fabry hemizygotes. All five patients in this report had no or extremely low α-Gal activity (0.6% or less) in lymphoblasts, and four of them showed phenotypic expressions of classic Fabry disease. Patient 3 was too young to be classified clinically. However, proteinuria was observed in his mother, and renal biopsy followed by pathological examination revealed changes in the glomerulus characteristic of Fabry disease.

A splice site mutation causing exon 4 skipping was previously reported in a Fabry patient (Yokoi et al. 1991), but the mutation site was different from that of ours: a g-to-a-substitution at the 3′ end of intron 3 in the previous report, and a G-to-T substitution at the 3′ end of exon 4 in our present study. The nonsense mutation in patient 2 has previously been described in another Japanese family (Sakuraba et al. 1990). At present, our patient is not known to be consanguineous with this family.

The missense mutations in our three patients were found in exon 3, although most of exonic point mutations in previous studies were in exons 1, 2, 5, 6, or 7. One of them (C142Y) expressed a considerable amount of enzyme protein in COS-1 cells with a complete loss of catalytic activity. This suggests a conformational change involving the active site in the enzyme molecule.

The other two missense mutations (A156V and L166V) expressed significant amounts of both catalytic activity and enzyme protein in COS-1 cells, although lymphoblast enzyme activities were not detectable. Previous expression studies in classic Fabry mutations revealed no detectable activity in transformed fibroblasts (Koide et al. 1990) or in COS-1 cells (Ishii et al. 1992). The expression products in this study were markedly thermolabile (more so at neutral pH). The mutant proteins are probably rapidly inactivated and degraded during transport to the lysosome through the endoplasmic reticulum and Golgi apparatus under neutral conditions.

In a few reports describing mutant gene products, α-Gal missense mutations causing classic Fabry disease result in the complete loss of catalytic activity. Our present study identifies two α-Gal gene mutants expressing catalytically active mutant enzymes that are rapidly inactivated in the cell in classic Fabry patients. We conclude that α-Gal gene mutations and expression are heterogeneous in Fabry disease. At least two groups have been identified in α-Gal missense mutants: one expressing a mutant enzyme without catalytic activity, and the other expressing a catalytically active but rapidly inactivated mutant enzyme. Further characterization of mutant proteins should lead to an understanding of its pathogenesis in more detail.

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ORIGINAL INVESTIGATION

Ans M. W. van den Ouweland · Mieke N. van der Est
Eveline Wesby-van Swaay · Ton S. L. N. Tijmensen
Frans J. Los · Jan O. Van Hemel · Raoul C. M. Hennekam
Hanne J. Meijers-Heijboer · Martinus F. Niermeijer
Dicky J. J. Halley

DNA diagnosis of Prader-Willi and Angelman syndromes with the probe PW71 (D15S63)

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Abstract Previously, 158 nuclear families with probands suspected of having either Prader Willi (PWS) or Angelman syndrome (AS) were analyzed with polymorphic DNA markers from the 15q11–13 region. These cases have been re-evaluated with the probe PW71 (D15S63), which detects parent-of-origin-specific alleles after digestion with a methylation-sensitive restriction enzyme (*Hpa*II). Application of PW71 to DNA samples isolated from leucocytes, confirmed the deletions and uniparental disomies detected earlier by marker analysis, and resolved 50% of the previously uninformative (n = 18) cases. PW71 and restriction fragment length polymorphism analysis indicated that, in all resolved cases, disomies of the 15q11–13 region were present. The use of PW71 increased the percentage of disomies detected in our PWS and AS patient groups. Almost 50% of our PWS patients and 17% of the AS patients showed a disomy of maternal or paternal origin, respectively. DNA of first trimester chorionic villi and of fibroblast cultures was not suitable for analysis with PW71 because of different methylation patterns. The application of PW71 is recommended for the diagnosis of the PWS and AS, with respect to DNA samples from blood.

Introduction

Patients affected by Prader-Willi syndrome (PWS) display a complex of developmental and neurological symptoms. The major features include characteristic facial features, e.g., almond-shaped eyes, severe hypotonia, failure to thrive, short stature, small hands and feet, hyperphagia with subsequent obesity, behavioral problems, hypogonadism, and mild to moderate mental retardation (Prader et al. 1956). The Angelman syndrome (AS) is characterized by mild hypotonia, absence of speech, inappropriate paroxysmal laughter, puppet-like jerky gait, microbrachycephaly, abnormal electroencephalogram (EEG), and severe mental retardation (Angelman 1965). However, the phenotype of patients with either PWS or AS does not always allow a firm diagnosis, especially at a young age.

PWS and AS are the archetypes of syndromes associated with genomic imprinting and both are attributable to abnormalities in the chromosomal region 15q11–13. In PWS, more than 60% of the patients show a molecular deletion of paternal origin in this region. Maternal disomy of chromosome 15 has been observed in 20%–25% of the PWS cases (Nicholls 1993). Familial cases of PWS are rare. Three familial cases showed no abnormalities in the 15q11–13 region using polymorphic markers (Örstavik et al. 1992; Reis et al. 1994). However, in one of these cases, a small deletion of the SNRPN gene was observed (Reis et al. 1994).

Deletions of the maternal chromosome 15q11–13 region have been observed in 70%–80% of sporadic AS patients, whereas a paternal disomy has been seen in only 3%–4% (Beuten et al. 1993; Nicholls 1993). In contrast to familial PWS, a deletion was described using polymorphic markers in one case of familial AS (Saitoh et al. 1992). Cytogenetic analysis of a family with both PWS and AS patients showed the presence of an unbalanced translocation t(15;22)(q13;q11) in the patients. As expected, the parental origin of the translocation chromosome determined the phenotype of the patients (Hultén et al. 1991). However, detectable abnormalities in familial AS cases are rare (Meijers-Heijboer et al. 1992; Wagstaff et al. 1992; Chan et al. 1993). Recently, sibs of two familial cases of AS were described who had inherited an apparently intact chromosome 15q11–13 region from both parents, excluding a detectable disomy or deletion of chromosome 15 (Glenn et al. 1993a; Reis et al. 1994). However, the methylation of the loci D15S9 and D15S63,

A. M. W. van den Ouweland (✉) · M. N. van der Est
E. Wesby-van Swaay · T. S. L. N. Tijmensen · F. J. Los
J. O. Van Hemel · H. J. Meijers-Heijboer · M. F. Niermeijer
D. J. J. Halley
Department of Clinical Genetics,
Erasmus University and University Hospital Dijkzigt,
P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

R. C. M. Hennekam
Department of Pediatrics and Institute for Human Genetics,
Academic Medical Center, Amsterdam, The Netherlands

both located in the 15q11–13 region, showed the same pattern as observed in AS patients with a deletion or disomy of the 15q11–13 region. In these siblings, the AS phenotype might therefore be the result of an altered imprinting process in the 15q11–13 region.

The isolation of several yeast artificial chromosomes clones located in the PWS/AS smallest region of deletion overlap (SRO), combined with the use of fluorescent in situ hybridization (FISH) analysis of chromosomes of PWS and AS cases with small deletions in the 15q11–13 region, has resulted in the separation of the PWS and AS critical regions (Kuwano et al. 1992). Several genes from this region have been isolated, a few of which are subject to genomic imprinting (Buiting et al. 1992; Cattanach et al. 1992; Leff et al. 1992; Nicholls 1993). The recently identified SNRPN gene is a good candidate gene for PWS. It has been shown that the SNRPN gene is expressed only from the paternally derived chromosome 15 in humans (Glenn et al. 1993b; Reed and Leff 1994). Recently, a deletion of the SNRPN gene has been identified in one sporadic and one familial case of PWS (Buiting et al. 1994; Reis et al. 1994). Furthermore, duplication of the maternal Snrpn region in mice results in an early postnatal lethality possibly associated with reduced sucking activity. This resembles the feeding difficulties and failure to thrive characteristic of PWS babies. In contrast, duplication of the paternal mouse gene is not associated with effects that might correspond to AS (Leff et al. 1992). Cattanach et al. (1992) have shown that a candidate gene for AS, GABRB-3, is not subject to imprinting in mice. Moreover, the GABRB-3 gene is not located in the SRO (Reis et al. 1993, 1994), and therefore this gene is no longer considered to be a candidate gene for AS.

Previously, we have performed molecular genetic analyses for 15q11–13 abnormalities in patients suspected of having PWS or AS, by means of restriction fragment length polymorphism (RFLP) and microsatellite markers. This procedure is laborious and not always informative. Probe PW71 (D15S63), located in the PWS critical region, allows the distinction of maternal methylated and paternal unmethylated alleles (Dittrich et al. 1992). We have used PW71 for additional studies on 85 suspected PWS and 73 suspected AS families. The comparative results show that PW71 is a useful probe in DNA diagnosis of patients suspected of having PWS or AS.

Patients and methods

Patients

A total of 85 families with a suspected PWS proband and 73 with a suspected AS proband were studied. Routine constitutional chromosome analysis was performed in 118 of the probands. DNA analysis was performed in these families to support the clinical suspicion of PWS or AS. In the case of suspected PWS, the only features known at referral were often mild to moderate mental retardation and obesity. In the case of suspected AS, patients exhibiting severe mental retardation combined with other features such as retarded speech, inappropriate laughter, epilepsy, microcephaly, or specific EEG characteristics were referred during diagnostic work-up by pediatricians, pediatric neurologists, and clinical geneticists.

DNA analysis

DNA from venous blood was isolated as described previously (Miller et al. 1988). Probes used in this study were: pML34 (D15S9), pTD3-21 (D15S10), pIR4-3R (D15S11), pTD189.1 (D15S13), pIR10-1 (D15S12), pCMW-1 (D15S24), and PW71 (D15S63). Microsatellite markers used were: IR4-3R, TD3-21, and GABRB3. Labeling of probes, hybridization, and radiolabeled polymerase chain reaction conditions were essentially as described (Dittrich et al. 1992; Meijers-Heijboer et al. 1992). All families were investigated using RFLP and microsatellite markers. Index patients of the families were also analyzed using the probe PW71. Genomic DNA was digested with the combination of the restriction enzyme *Hin*dIII and the methylation-sensitive restriction enzyme *Hpa*II; Southern blots were hybridized with the probe PW71. FISH with probe c189.1 (R.D. Nicholls, personal communication) was applied on lymphocyte metaphase spreads as described by Kievits et al. (1990).

Results and discussion

Comparison of the results of RFLP and microsatellite analysis with methylation analysis using the probe PW71

Until recently, only RFLP and microsatellite markers were available for the molecular genetic evaluation of PWS and AS families. The results of these analyses in 85 and 73 families with probands suspected of PWS and AS, respectively, are summarized in Table 1. PWS was confirmed in 24 probands; 16 of them showed a paternal deletion with the markers and, in 8 patients, a maternal disomy could be detected. All these PWS patients showing a deletion or disomy based on RFLP and microsatellite markers also exhibited the absence of the unmethylated paternal allele detected by PW71 (Table 1). Fifty probands had chromosome 15 alleles from both parents and the diagnosis of PWS remained in doubt. DNA of these PWS patients exhibited a maternal and a paternal allele in the screening with the probe PW71. RFLP/microsatellite analysis was uninformative in 11 families (13% of the total number of PWS families). Of these 11 families, six index patients did not show a paternal allele after analysis with PW71, indicating PWS in the patients. In these six cases, it had not been possible to distinguish between biparental inheritance and a maternal disomy using the RFLP and microsatellite markers. Because of the absence of a paternal allele with probe PW71, we concluded that, in these six cases, the observed RFLP pattern is the result of a maternal disomy. This highlights the value of PW71 analysis in detecting abnormalities in PWS patients.

Of the suspected PWS probands with a normal PW71 pattern (n = 55), further clinical examination indicated that four patients fulfilled the clinical criteria for PWS as proposed at the international conference on Prader-Willi syndrome (1991), with no detectable abnormality with the presently available DNA analysis. One other family had two sibs initially suspected of having PWS. Both sibs

Table 1 Re-evaluation of suspected PWS and AS cases with probe PW71 (D15S63)

	n	PW71*
Prader-Willi syndrome (*n* = 85)		
Informative families using RFLP/microsatellite markers (n = 74)		
Deletion	16	M
Disomy		
Isodisomy	3	M
Heterodisomy	5	M
Biparental inheritance	50	MP
Uninformative families using RFLP/microsatellite markers (n = 11)		
Deletion	0	
Disomy		
Isodisomy	2	M
Heterodysomy	3	M
Iso- or heterodisomy	1	M
Biparental inheritance	5	MP
Angelman Syndrome (*n* = 73)		
Informative families using RFLP/microsatellite markers (n = 66)		
Deletion	20	P
Disomy		
Iso- or heterodisomy	1	P
Biparental inheritance	44	MP
Biparental inheritance	1	P
Uninformative families using RFLP/microsatellite markers (n = 7)		
Deletion	0	
Disomy		
Heterodisomy	2	P
Iso- or heterodisomy	1	P
Biparental inheritance	4	MP

[a] *M* methylated maternal allele present, *P* unmethylated paternal allele present

showed the same paternal haplotype in the critical region. The paternal contribution included the unmethylated fragment detected by PW71. Additional clinical study confirmed the Cohen syndrome. After questioning the diagnosis of PWS by DNA results, further diagnostic studies in this group resulted in diagnoses of the Cohen syndrome, the Opitz Frias syndrome, the Bardet-Biedl syndrome, the fragile X syndrome, and the Albright syndrome. One patient with a mosaic tetraploidy was also observed.

Twenty patients were definitively diagnosed as having AS by the identification of maternal deletions using RFLP and microsatellite analysis. These deletions were confirmed with the probe PW71 (see Table 1). In one case, a paternal disomy had been strongly suggested by the presence of only non-maternal alleles in the proband by using RFLP and microsatellite markers on the DNA of the proband and her mother. In this AS patient, FISH analysis

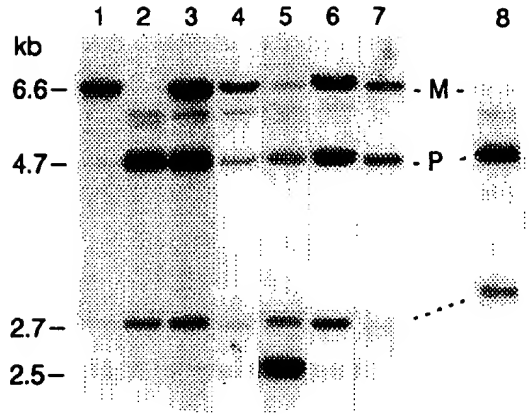

Fig. 1 Southern blot analysis using PW71 (D15S63) as a molecular probe. Double digested *Hin*dIII+*Hpa*II genomic DNA was electrophoresed, blotted and hybridized with probe PW71. *Lane 1* Leucocytes from a PWS patient (previously shown to have a deletion), *lane 2* leucocytes from an AS patient (previously shown to have a deletion), *lane 3* leucocytes from a normal control, *lane 4* fibroblast cell line of a PWS patient (known to exhibit a deletion), *lane 5* chorionic villi from a fetus affected by fragile X syndrome, *lane 6* cultured amniotic fluid cells, *lane 7* abortion material of the fetus affected by fragile X syndrome (see also *lane 5*) *lane 8* leucocytes from an AS patient (biparental mode of 15q11–13 inheritance), *M* methylated maternal allele, *P* unmethylated paternal allele

using the cosmid c189.1 as a probe resulted in signals on both chromosomes 15, and PW71 detected only the unmethylated paternal allele. In 44 cases, a maternal allele could be identified, both with RFLP/microsatellite analysis and with probe PW71, including the large AS family described by Meijers-Heijboer et al. (1992). In this AS family, the AS phenotype is clearly linked to chromosome 15q11–13 markers. No deletions or disomies and a normal PW71 pattern were observed in AS patients of this family.

One phenotypically "typical" AS patient had a normal biparental mode of inheritance with 15q11–13 markers, but a lack of the methylated maternal fragment using PW71 (Fig. 1, lane 8). The patient's phenotype included severe mental retardation, microbrachycephaly, large chin, ataxia, and restlessness. Outbursts of inappropriate laughter were found only infrequently, however, and she had never had any fits. Sample switches were excluded. Southern blot analysis of genomic DNA of this patient digested with *Hin*dIII (and not *Hpa*II) indicated that there was no deletion of the D15S63 locus, supporting the conclusion that a class of AS patients has a normal biparental mode of inheritance of the 15q11–13 region but an abnormal methylation pattern in this region. Familial cases of AS and sporadic patients with the same phenomenon have recently been described (Glenn et al. 1993a; Reis et al. 1994).

In seven cases of suspected AS, DNA marker analysis was inconclusive. Analysis with PW71 showed a paternal disomy in one case, whereas in the other cases, a biparental inheritance was observed. In two cases, DNA of the index patient and only one of his parents was available. In these cases, two different alleles could be ob-

served with several 15q11–13 markers, whereas PW71 detected a paternal allele only (data not shown). These patterns are probably the result of a paternal heterodisomy, but this could not be confirmed because of lack of material from the other parent. Alternatively, these results could also reflect a defect in the methylation mechanism of the 15q11–13 region (Glenn et al. 1993a; Reis et al. 1994).

Of the suspected AS probands (n = 48) exhibiting a normal PW71 pattern, nine patients were affected with AS according to the criteria described by Clayton-Smith and Pembrey (1992). Further clinical examination indicated other diagnoses in the remaining group of patients (n = 39), including microcephaly, midline-cleft, Peters-Plus syndrome, Rett syndrome, X-linked mental retardation, Smith-Lemli-Opitz syndrome, and Opitz trigonocephaly.

Cytogenetic versus DNA analysis

Data of routine constitutional chromosome analysis were available for 70 out of 85 suspected PWS probands and for 48 out of 73 suspected AS probands. Cytogenetic analysis showed deletions in the chromosome 15q11–13 region in 10 PWS patients and deletions in seven AS patients. Five of these 17 deletions could not be confirmed by DNA analysis (RFLP/microsatellite markers and PW71). Re-evaluation using FISH analysis of one of these five cytogenetic deletions with the probe c189.1 resulted in signals on both chromosomes 15. A similar experience was reported by Chan et al. (1993) who showed that, in five cases, deletions were cytogenetically detected but could not be confirmed at the molecular level. In our group, of the 118 cytogenetically tested patients suspected of PWS or AS, 29 showed a deletion at the DNA level. Of these 29 molecularly detected deletions, only 12 (41%) were identified using chromosome analysis. Our data using the PW71 probe indicated that, at least in our group of patients, a high percentage of disomies was present, even up to about 50%. Therefore, molecular analysis must be performed to identify abnormalities in the 15q11–13 region for the diagnosis of PWS and AS. However, routine chromosome analysis must still be performed to identify other chromosome abnormalities, including translocations and ring chromosomes. Glenn et al. (1993a) described two atypical PWS patients exhibiting a biparental mode of inheritance of the 15q11–13 region. In these patients, a normal PW71 pattern but an altered methylation at D15S9, approaching a typical PWS pattern, was observed. Both patients have a ring chromosome 15 of paternal origin. Moreover, a ring chromosome 15 was observed in one of our suspected PWS patients with a normal PW71 and a biparental inheritance pattern. This patient showed hyperphagia, obesity, short statue, small hands and feet, almond-shaped eyes, late onset of menstruation, mental retardation, strabismus, and hypothyroidism, representing several clinical criteria of PWS. The parental origin of this ring chromosome 15 and the methylation status of the D15S9 locus are still a subject of investigation.

Probe PW71 to screen DNA isolated from different tissues

Sometimes, cultured fibroblasts of an index case are the only available source for DNA isolation. The pattern obtained with probe PW71 in DNA isolated from fresh blood leucocytes was compared with one fibroblast strain from a PWS patient and seven control fibroblast strains. The PWS patient has a deletion demonstrated by RFLP analysis, but PW71 showed a reduced intensity of a normal-sized paternal allele (Fig. 1, lane 4). In DNA isolated from control fibroblast cell strains hypomethylation of the region was observed using probe PW71 (data not shown). A pattern of hypomethylation had also been observed in several solid tumors and placenta by Dittrich et al. (1993). In conclusion, DNA isolated from cell strains and some tissues is not suitable for analysis with PW71.

To investigate whether PW71 is useful for prenatal analysis, DNAs of several chorionic villi samples from pregnancies at risk for different genetic human diseases were tested. A reduction of the maternal and the paternal fragments was observed in all cases tested, whereas a clearly hybridizing smaller fragment (2.5 kb) appeared, indicating that this region is hypomethylated in DNA isolated from chorionic villi. A typical example is given in Fig. 1, lane 5. Fetal tissue obtained from the fetus affected by fragile X syndrome, after termination at 14 weeks of gestation, showed a normal methylation pattern (Fig. 1, lane 7). The methylation at the D15S63 locus was also tested in DNA isolated from cultured amniotic fluid cells (Fig. 1, lane 6); methylated maternal and unmethylated paternal fragments of the same size as those in DNA from blood leucocytes were found. The hypomethylation observed in chorionic villi and the normal pattern in cultured amniotic fluid cells are in agreement with the recent data of Dittrich et al. (1993). The results obtained with fetal DNA show that chorionic villi are not a suitable source for DNA for PW71 analysis. Hence, this probe cannot be used for first trimester prenatal diagnosis of PWS and AS. Apparently, the methylation status at the D15S63 locus resembles that of the CpG island of the FMR-1 gene, the gene underlying fragile X syndrome. In prenatal diagnosis of the fragile X syndrome, the *Eco*RI/*Eag*I methylation assay cannot be performed using DNA isolated from chorionic villi, in contrast to DNA isolated from amniotic fluid cells and other fetal tissues (Sutherland et al. 1991; Sutcliffe et al. 1992).

Concluding remarks

Re-evaluation with the probe PW71 of nuclear families with probands suspected of having either PWS or AS resulted in an increase in the number of disomies detected in PWS and AS families. In 35.3% of the suspected PWS cases, abnormalities in the PWS region could be detected. In addition, a number of other diagnoses were established after molecular exclusion of detectable abnormalities in the 15q11–13 region and extended clinical evalutation. In